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Enhancement of HIF-1-mediated Tumor Radiosensitization via Vascular Normalization

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HST.525J - Tumor Pathophysiology and Transport Phenomena: A Systems Biology Approach

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In this proposal, the combination of vascular normalization with HIF-1 blockade is suggested as a possible means to radiosensitizing tumors. Research has shown that, after administering antiangiogenic agents, there is window of vascular normalization in which tumor hypoxia is alleviated, and tumor radiosensitivity is increased. Recent research also shows that HIF-1 blockade after fractionated radiotherapy causes significant tumor vascular radiosensitization. The careful combination of vascular normalization with post-irradiative HIF-1 blockade has the potential to further increase tumor radiosensitivity through the interplay of the effects of both therapies. It is hypothesized that the partial tumor reoxygenation, due to vascular normalization, will restrict the radioprotective effects that HIF-1 inhibition has on distal tumor cells. It is also hypothesized that partial tumor reoxygenation might allow a greater magnitude of HIF-1 activation upon irradiation. This would make the administration of HIF-1 inhibition following the irradiation of a vasculature-normalized tumor a promising method of further radiosensitizing tumors.

1. SPECIFIC AIMS

The importance of developing effective cancer treatment protocols is increasingly important, as cancer mortality has remained relatively constant over the past few decades. In contrast, the mortality of other diseases, such as heart disease, cerebrovascular disease, pneumonia, and influenza, has dropped by more than 50% in each case. However, current treatments of cancer can be improved significantly, through the application of relatively new concepts in tumor biology. The general goal here is to apply some of those concepts to radiation therapy, in hopes of significantly sensitizing tumors to fractionated radiation therapy, while not affecting the radiosensitivity of normal tissue. In pursuit of this general goal, the aim here is to determine whether the brief window of vascular normalization, as caused by antiangiogenic agents, augments the tumor radiosensitivity caused by HIF-1 inhibition, and vice-versa.

1.1. Effect of the Combination of Vascular Normalization and HIF-1 Blockade on the Outcome of Radiotherapy

It is hypothesized here that combination of changing the tumor microenvironment (through vascular normalization), irradiating the tumor, and afterwards administering a HIF-1 inhibitor, will show a greater than additive effect on delaying tumor growth. Previous work has shown that monoclonal antibody to vascular endothelial growth factor receptor 2 (VEGFR-2) enhances tumor radiosensitivity during a specific window of time corresponding to vascular normalization [16]. Other work has shown that hypoxia-inducible factor 1 (HIF-1) plays an important role in the radioprotection of tumor vasculature in response to fractionated radiotherapy [14]. HIF-1 blockade therefore sensitizes the tumor vasculature to ionization radiation. However, the effect of HIF-1 blockade *during* the above mentioned window of vascular normalization has not been explored. If the hypothesis stated above is correct, and a greater-than-additive effect is on tumor growth delay is observed, then it is hypothesized that two specific mechanisms account for some or all of the observed effect.

1.2. Hypothesized Mechanisms Accounting for Extended Delay in Tumor Growth

One possible cause of greater-than-additive radiosensitizing effect of of the combination discussed above is that vascular normalization will reduce the radioprotective effect that HIF-1 blockade has on distal tumor cells. While that hypothesis is difficult to test, it can be inferred from the

research done by Moeller and colleagues ([20], see section 2.2 and 2.3).

Another prevailing hypothesis in this experiment is that because vascular normalization relieves tumor hypoxia, the magnitude of HIF-1 activation due to irradiation is greater in vasculature-normalized tumors than it is in more hypoxic, untreated tumors. This hypothesis of arises from the fact that vascular normalization reduces, but does not eliminate, hypoxia in the tumor microenvironment [17]. After normalization, the increased oxygenation would likely allow for the enhanced generation of reactive oxygen and reactive nitrogen species (ROS and RNS) upon irradiation. This would increase the level of oxidative stress in the post-irradiative tumor microenvironment, and effectively stabilize the active form of HIF-1 [14]. Note that in this model, the concentration of ROS and RNS in a post-irradiative, vascular-normalized tumor would be greater than the same in a post-irradiative, unaltered tumor. Thus, HIF-1 blockade after vascular normalization and radiation therapy should significantly affect the treatment outcome in a positive way. An aim of the experiments described in this proposal is to determine if this is indeed the case.

2. BACKGROUND AND SIGNIFICANCE

2.1. Cancer as a Major Disease

Cancer is the second leading cause of death in the United States, accounting for 23% of all deaths in the country. The lifetime probability of getting cancer is nearly 50% for men and 33% for women. For children, the probability of getting cancer between the ages of 0 and 14 is 15%. While the mortality of the disease varies significantly depending on the type of cancer, the time of diagnosis, and patient demographic, the treatments currently available for all types of cancers are fairly unsophisticated. They are not wholly specific for cancer tissue microenvironments and they, in general, do not have spectacular cure rates. Furthermore, cancer survival rates have not improved appreciably in the past 50 years (all statistics from The American Cancer Society [1]). As cancer awareness rises and diagnostic methods improve, the need for more effective cancer treatments is becoming more and more emphasized.

Currently, there are several different options for cancer treatment, and most cancer patients undergo some combination of the treatments available.

2.2. Current Treatment Options

Surgery is a procedure performed on nearly all cancer patients as a diagnostic procedure. It is also used as a treatment; when it is, it is usually accompanied by radiation therapy and/or chemotherapy. The objective of a surgical treatment of cancer is to remove as much of the primary tumor as possible, and then allow ionizing radiation or chemotherpeutic agents to kill the remaining tumor cells. The primary drawback of surgery is that is invasive, it (in some cases) results in physical abnormalites, and it also involves the side-effects of therapies that it is combined with.

Radiation Therapy is a very common form of treatment in which tumors are targeted and irradiated by a source of ionizing radiation (IR); approximately half of all cancer patients receive some form of the treatment. It was thought that the primary action of radiation therapy was to cause mutations in the DNA of the irradiated cells, thereby inducing apoptosis during the replication phase of the cells' lives. However, recent research suggests that the apoptotic effects of IR are due to intercellular signaling from irradiated endothelial cells [5] (see section 2.2). Radiotherapy is administered externally (via an IR producing machine) or internally (via implanted sources of IR), depending on the type of tumor being treated. A significant disadvantage of radiation therapy is that it also damages the normal tissue surrounding the tumor. A large amount of research is being done on various tumor-radiosensitizers and normal tissue-radioprotectors.

Chemotherapy is the administration of cytotoxic drugs that target and cause lethal damage to cells undergoing mitosis. Because cancer cells are dividing much more rapidly than most other cells in the body, chemotherapeutic drugs are particularly effective against tumors. However, these drugs can also affect normal, rapidly dividing cells, including bone marrow cells and cells of the gastrointestinal tract. Thus, determining the correct dosage is very important, and will depend on both the tumor and the patient.

Other, more modern anti-cancer therapies also exist, though most of them are in clinical trials, or being researched heavily. These therapies include antiangiogenic therapy, immunotherapy, and photodynamic therapy (PDT). Of these options, antiangiogenic therapy has received the most attention, as it has shown significant promise when combined with radiotherapy and chemotherapy. The first antiangiogenic drug, Avastin (bevacizumab) has already been approved for use with chemotherapeutic agents.

Combination Therapies are the most effective forms of cancer treatment availablie. Such therapies use multiple treatment modalities to eliminate tumors. In fact, nearly all cancer patients today receive some form of multi-modal therapy. The combination of surgery and radiation therapy

was already mentioned above, but chemotherapy is also often used in conjunction with radiation therapy and/or surgery. In the case of chemotherapy, the cytotoxic drugs used are not as effective at the center of a tumor as they are on the tumor periphery. This is partly due to the abnormal vasculature and the interstitial environment of a tumor, both of which prevent chemotherapeutic agents from readily diffusing to the center of the tumor [2]. However, it is also due to the fact that cells at the center of a tumor are not dividing as rapidly as cells at the periphery. Thus, to effectively eliminate a tumor, chemotherapy is often combined with one or even two different treatments. In combination therapies, antiangiogenic drugs show significant promise. As will be discussed below, these drugs can be used to radiosensitize tumors, and also to "fix" abnormal tumor vasculature prior to the administration of other anti-cancer drugs.

2.3. Action of Radiation Therapy

As mentioned above, the accepted mechanism of action of radiation therapy has changed in recent years. Several studies have suggested that radiation therapy, when combined with the administration of antiangiogenic drugs, results in a greater than additive increase in the effectiveness of the anti-tumor radiotherapy [3, 5, 7]. These studies revealed a link between the tumor and its microvasculature that was previously unknown.

Recent research suggests that radiation therapy causes damage to tumors by targeting tumor microvasculature, rather than tumor cells [10]. The prevailing hypothesis before this research held that permanent radiation damage to tumors resulted from reproductive cell death, caused by direct, cytotoxic irradiation of tumor stem cell clonogens. However, it has been shown that, after single-dose irradiation of a tumor, the endothelial cells (ECs) of the microvasculature induce surrounding cells to apoptize via ceramide and ASMase signaling [13, 19]. For example, in gastrointestinal studies [13, 19], it has been shown that, when ceramide signaling is enhanced by the inhibition of ATM kinase (a repressor of ceramide synthase), post-irradiative stem cell clonogen survival is significantly decreased. This EC-based sensitization of crypt stem cells is supported by other GI experiments [8]: pretreatment of cells with bFGF reduces EC apoptosis, and thus increases stem cell clonogen survival; deletion of Smpd1, a gene ecnoding the apoptosis mediating factor ASMase, also results in attenuation of EC apoptosis (and stem cell clonogen death). While ceramide and ASMase signaling play very important roles in the response of a tumor to single-dose radiotherapy, Moeller et al. revealed another important factor in the regulation of tumor response to fractionated radiotherapy.

In recent studies, Moeller et al. [14] showed that fractionated radiotherapy induces a radioprotective effect on the tumor vasculature through the action of HIF-1. In particular, they were able to link radiation-induced tumor reoxygenation to HIF-1 activation, though HIF-1 is generally thought to be activated by hypoxia. Moeller et al. showed that, upon exposure to radiation, the concentration of ROS in the tumor rises, causing an increase in the oxidative stress of the tumor microenvironment. This oxidative stress, though accompanied by tumor reoxygenation, is sufficient to stabilize the active form of the HIF-1α subunit. In its stable form, HIF-1α binds to hypoxia-response elements (HREs) and stimulates the expression of several downstream genes that control tumor metabolism, growth, and angiogenesis. However, this is not the only mechanism through which irradiation induces HIF-1 mediated cell-survival signaling. The studies of Moeller et al. also revealed the intracellular presence of stress-granules that hold HIF-1 mRNA transcripts. As the tumor environment becomes hypoxic, HIF-1 transcripts are trapped and stored in stress-granules. When the hypoxic tumor is exposed to ionizing radiation, reoxygenation occurs, and the transcripts are released and transcribed. As more HIF-1 is translated, the transcription of several HRE-controlled cell-survival factors, including VEGF, is stimulated.

In summary, HIF-1 is an important regulator of the tumor response to IR [14]. Further adding to the its potential significance in cancer therapy, HIF-1 is also an important regulator of cellular apoptosis, proliferation, and angiogenesis [4]. Though the regulatory roles of HIF-1 already make the protein important very relevant to the current proposal, a very interesting possibility arises when one considers the fact that HIF-1 is responsive to certain characteristics in the tissue microenvironment that are known to differentiate normal tissue from cancerous tissue (*e.g.* tissue oxygenation). In particular, HIF-1 is active in cancerous tissue, where it promotes cytokine-based cell-survival signaling, and it is inactive in normal tissue. Thus, as hypothesized by Moeller et al., HIF-1 inhibition might significantly radiosensitize tumor cells while leaving the cells of normal tissue unaffected.

2.4. HIF-1 Blockade Affects Tumor Radiosensitivity

Following up on their hypothesis, Moeller and colleagues tested the effects of HIF-1 blockade on tumor radiosensitivity [20]. Because the role played HIF-1 is very complex role and involves the regulation several different phenotypes, Moeller et al. [20] sought to determine the overall effect of HIF-1 blockade on the radiosensitivity of tumor cells and tumor vasculature. They found that HIF-1 promotes tumor vessel radioresistance, and that HIF-1 inhibition dramatically increases tumor

vasculature radiosensitivity, regardless of whether inhibition occurs before or after irradiation. With regard to central tumor cells, which are hypoxic and have slowed metabolism, HIF-1 acts to maintain glucose metabolism and ATP production - thus, the inhibition of HIF-1 decreases central tumor cell bioenergetics. In fact, this effect is significant enough that HIF-1 inhibition ends up protecting central tumor cells from ionizing radiation through causing a reduction in their cellular activity. Furthermore, the Moeller et al. research suggests that HIF-1 promotes cell cycle arrest in hypoxic tumor cells that have access to glycolytic energy stores, indicating that HIF-1 inhibition might radiosensitize these cells through encouraging post-irradiative mitosis. In cells that are both glucose and oxygen starved, HIF-1 increases cellular radiosensitivity by sustaining mitotic rates; consequently, HIF-1 inhibition would be radioprotective in these "distal" tumor cells. As it can be seen, the ultimate effect of HIF-1 blockade on cellular radiosensitivity is highly dependent on the surrounding microenvironment.

In conducting the research described above, Moeller et al. also found that the effect that HIF-1 inhibition has on tumor cells is primarily due to the cells' immediate response to irradiation. Therefore, administering HIF-1 inhibitor after exposing a tumor to IR would minimizing the possible radioprotective effects on the tumor interior. In contrast, such timing would not change the effect that HIF-1 inhibition has on the tumor vasculature (as mentioned above). Thus, Moeller et al. recommend a radiation-first approach to augmenting radiotherapy with HIF-1 inhibition. However, this conclusion is based on experiments performed with constant, unaltered tumor microenvironments.

2.5. Alteration of Tumor Microenvironment Through Vascular Normalization

A relatively new concept in cancer biology is the normalization of a tumor's vasculature in response to antiangiogenic drugs. Conventional belief regarding antiangiogenic therapy is that the administration of angiogenesis inhibitors results in the destruction of a tumor's vasculature. Consequently, the tumor is starved of oxygen and nutrients and cannot grow or survive. However, recent research suggests that, in the course of destroying tumor vasculature, certain antiangiogenic agents can actually cause the vasculature to become more "normal" and efficient at delivering nutrients for a brief period of time [17]. In brain tumors, this was shown to happen through increased pericyte coverage and a corresponding activation of matrix metalloproteinases [16]. The transient normalization provided by antiangiogenic agents can alleviate the hypoxic environment of tumors and also allow for improved drug delivery to the tumor interior. Thus, it has become

increasingly apparent that the window of normalization provided by antiangiogenic agents, such as VEGFR-2 antibody and thalidomide, can be used to optimize the response of tumors to cytotoxic therapies [15, 18]. Because vascular normalization alters the microenvironment of a tumor, it might also help to maximize tumor radiosensitivity in response to HIF-1 blockade.

2.6. Vascular Normalization combined with HIF-1 Blockade

As mentioned earlier, the effect of HIF-1 on tumor cells is highly dependent on the surrounding tumor microenvironment. To summarize, HIF-1 enhances tumor radiosensitivity in nutrient-starved, distal tumor cells through promoting apoptosis, metabolism, and proliferation of these cells. However, in the tumor vasculature, HIF-1 decreases radiosensitivity through cytokine-mediated cell protection [20]. Many of the experiments performed by Moeller et al. in investigating the pleiotropic effects of HIF-1 blockade involved *in vitro* cell cultures. While the experiments do yield significant insight into various effects of HIF-1 blockade, they do not readily suggest what might happen in changing tumor microenvironments *in vivo*.

One way to alter the *in vivo* tumor microenvironment is to normalize the tumor vasculature. As discussed above, this can be done through careful application of antiangiogenic therapy. In combining this approach with HIF-1 blockade, it is hypothesized that the alleviation of hypoxia resulting from vascular normalization will decrease the radioprotective effects that HIF-1 inhibition has on the distal tumor cells. It is also hypothesized that the increased tumor oxygenation prior to radiotherapy will result in an increased level of oxidative stress in the post-irradiative tumor microenvironment. This stress would stabilize the active form of HIF-1, making HIF-1 inhibition all the more useful when a vasculature-normalized tumor is treated with fractionated radiotherapy.

As mentioned in section 1.2, the hypothesis regarding oxidative stress arises from the fact that vascular normalization reduces, but does not eliminate, hypoxia in the tumor microenvironment [17]. Upon normalization, the resultant increase in pO_2, relative to the unaltered tumor microenvironment, would amplify the generation of superoxide radicals ($^{\bullet}O_2^-$) in response to radiotherapy. An increase in $[^{\bullet}O_2^-]$ would cause the chemical creation of other ROS and RNS to rise [12], thereby increasing the level oxidative stress in the tumor microenvironment. Because the active form of HIF-1 is stabilized by oxidative stress [14], one might expect a greater magnitude of radiation-induced HIF-1 activation in vascular-normalized tumors than in unaltered tumors.

3. RESEARCH DESIGN AND METHODS

The effect of vascular normalization on tumor radiosensitization via HIF-1 inhibition will be explored. Human tumors will be grown in mice, and different methods of HIF-1 blockade will be tested. Vascular normalization will be achieved through the administration of a VEGFR-2 antibody, as done in the work of Winkler et al. and Tong et al.[15, 16]. HIF-1 blockade will be achieved through the use of YC-1, as done in the work of Moeller et al. [14].

3.1. Experiment Setup

A single tumor cell line, orthotopic U87 MG (human glioblastoma), will be used in these experiments. The U87 human glioblastoma cell line has been shown to exhibit vascular normalization in response to VEGFR-2 antibody [6, 15, 16]. The tumors will be subcutaneously injected into immunocompromised (SCID) mice, and xenografts will be grown for experimentation.

HIF-1 inhibition will be achieved through means similar to those of Moeller et al. [14, 20]. In particular, the HIF-1 targeting drug YC-1 [9] will be used to inhibit HIF-1 activity. However, this drug will have to be characterized, and its use will have to be validated in the experiments to be performed. Because these experiments are meant to be somewhat similar to actual therapeutic protocols, mutant HIF-1 negative cell lines will not be used.

Vascular normalization will be achieved through DC101, a VEGFR-2 monoclonal antibody. DC101 will be administered to a given group of mice for a given period of time, and radiation therapy and HIF-1 blockade will be administered at the end of these time periods. A number of mice in each group will not be administered DC101; they will serve as controls, and as non-treated mice for other experiments. This organization will allow for a range of vascular regression, and hence a range of tumor microenvironments, to be tested with HIF-1 inhibition and IR. Using Winkler et al.[16] as a preliminary guide, DC101 will be administered to mice in quantities of 40 mg/kg. This dosage will need to be validated and altered as needed.

3.2. Determining the Effect of the Proposed Treatment Combination

Several experiments will need to be performed to come to a conclusion regarding the effect of vascular normalization on HIF-1 based radiosensitization of tumors. The results of the experiments proposed in this section will help to accept or reject the hypothesis of section 1.1. As mentioned before, mice will be divided into several different treatment groups based on the day that the mice

will be analyzed. For example, group 0 would represent mice to be analyzed on day 0, group 1 would represent mice to be analyzed on day 1, and so on. A window of 10 days following DC101 treatment will be observed, following Winkler et al.

1. Control mice (not treated with DC101) will be left alone for baseline tumor growth analysis.

2. Untreated mice xenografts from each group will be treated with fractionated radiotherapy. The resultant effect on the implanted tumors will be compared to the results of the following experiments. In general, tumor growth should be delayed.

3. Untreated mice from each group will be treated with HIF-1 inhibitor. The effect of HIF-1 blockade alone on tumor growth will be observed.

4. Untreated mice from each group will be further divided into two subgroups. The first subgroup will be treated with HIF-1 inhibitor prior to fractionated IR therapy, and the second subgroup will be treated with the inhibitor after radiotherapy. This experiment will allow validation of the mice against the results of Moeller et al. For validation, results should show that HIF-1 inhibition is maximally radiosensitizing if the inhibitor is administered after radiotherapy.

5. Mice from each group will be treated with DC101. The effect on tumor volume, as caused by the VEGFR-2 antibody alone, will be measured.

6. DC101 treated mice from each group will be exposed to fractionated IR *without* HIF-1 inhibition. The results of this study will allow validation of the xenografts against the results of Winkler et al. For validation, the effect of combined radiation therapy and DC101 treatment should cause a greater than additive tumor growth delay in mice that have been treated with DC101 for 4 to 6 days. All other groups should show a no more than additive tumor growth delay.

7. DC101 treated mice from each group will be exposed to HIF-1 inhibitor *without* radiotherapy. This experiment will be used to determine if the effects of HIF-1 inhibition are coupled to radiotherapy - it is expected that this will be the case, since HIF-1 activation is largely dependent on the radiation-induced microenvironment.

8. DC101 treated mice from each group will be exposed to fractionated IR and HIF-1 inhibition. Here, as in the fourth experiment, HIF-1 inhibition will be administered before radiotherapy

in some mice, and after radiotherapy in others. This is the final experiment needed to test the hypothesis that vascular normalization, fractionated radiotherapy, and HIF-1 inhibition produce a greater-than-additive delay in tumor growth.

In this series of experiments, each of the three treatment variables (VEGFR-2 antibody, fractionated IR therapy, HIF-1 inhibitor) is tested in every possible combination with the other variables. The results from these experiments will determine if the effect of combining vascular normalization with HIF-1 inhibition results in a greater-than-additive sensitization to IR. The experiments will allow for a comparison of the model being used against previously published results. They will also provide the data necessary to determine if any of the observed effects are due to the coupling of two of the three treatments, rather than the hypothesized interplay of all three.

In addition to measuring the effect of the various treatment combinations on tumor growth delay, tumor oxygenation, perfusion, and interstitial fluid pressure (IFP) will be measured. The purpose of these measurements is to check if vascular normalization is occurring, and if the tumor microenvironment is changing as a result. Vascular normalization should result in an observable increase in tumor pO_2 and vascular perfusion, and a decrease in IFP. In order to obtain this data, a measurement protocol similar to that of Ansiaux et al. [18] will be followed:

- Electronic paramagnetic resonance (EPR) oximetry will be used to measure changes in tumor oxygenation.
- Tumor perfusion will be measured using dynamic contrast-enhanced magnetic resonance imaging.
- IFP will me measured using a polyurethane transducer-tipped catheter [21]. This method should allow for simple measurement of tumor IFP. However, the standard wick-in-needle approach may prove to be a better option, due to its widespread use and acceptance.

Through the measurement of these variables in the 8 treatment models described above, the effect the combination of vascular normalization with HIF-1 inhibition can be determined, and it will be possible to attribute this effect to specific changes in the tumor microenvironment.

3.3. Determining the Potential Mechanisms Causing the Observed Effects

In these experiments, the effect of the various treatments on HIF-1 activation and the creation of ROS and RNS will be examined more carefully. The results of the following experiments will

help accept or reject the hypotheses of section 1.2.

In one experiment, HIF-1 activation will also be examined through the use U87 cells that have been transfected with with a GFP reporter of HIF-1 activity. In these cells, the gene for GFP will be under the control of an HRE. The HRE is activated by the binding of active HIF-1. Such an experiment would help to determine how much vascular normalization alters HIF-1 activation prior to radiation therapy. The transfection and measurement protocol would follow that of Moeller et al. [14].

To determine if the level of post-irradiative ROS and RNS generation is indeed enhanced in vascular normalized tumors, the concentration of these species will be measured in control mice, DC101 treated mice, control mice treated with IR, and DC101 mice treated with IR. The level of ROS and RNS in the tumor tissue will be determined using the assay developed by Moeller et al. [14]. In this assay, carboxy-2 dichlorofluorescein diacetate (H_2DCFDA), a free radical-sensitive dye, is used to saturate skinfold window chamber tumors prior to irradiation. H_2DCFDA reacts with free radical species to become the fluorescent DCFDA. Controls will be used following Moeller et al. In particular, DCFDA will be used to saturate control tumors, and the effect of radiation on dye accumulation will be controlled for. To ensure that free radicals are the primary species reacting with H_2DCFDA, Moeller et al. use a superoxide dismutase (SOD) mimetic of low molecular weight to inhibit the free radical-induced conversion of H_2DCFDA to DCFDA. This will be done here as well, in order to validate the use of H_2DCFDA in the U87 xenografts.

3.4. Potential Pitfalls

In every research endeavor, there is the possibility that some hypothesis will not hold, or that some proposed experiment simply will not work. Some of these possibilities have been speculated on below, and likely responses to them have been determined:

- There may be errors in the proposed measurement protocol. If there are, other methods will be pursued, and other cell lines can also be pursued. There are at least 2 different methods for each of the major measurements listed in the previous section. Tumor oxygenation can be measure using a luminescence fiber-optic sensor, tumor perfusion can be measured using laser doppler imaging and dye staining, and IFP can be measured using the standard wick-in-needle method.

- Vascular normalization might reoxygenate the tumor enough such that HIF-1 inhibition becomes inconsequential. If this scenario is possible, precise timing would have to be used to perform the experiments before the tumors become too oxygenated. In this case, a simple, multi-use oxygenation sensor, such as the fiber-optic sensor mentioned above, would be useful. An expansion on this scenario is presented last.

- The effect of vascular normalization on radiation-induced ROS and RNS might be over-estimated or over-valued. In this case, we [23] would count on the hypothesis that the tumor reoxygenation would at least render the distal tumor cells less prone to being radioprotected by HIF-1 inhibition. If this is also not the case, and both of the hypotheses are incorrect, then we would hope that an additive effect is observed.

- In the worst case scenario, as mentioned above, it is possible that vascular normalization will reoxygenate the tumor enough so that the benefits of HIF-1 inhibition are lost due to HIF-1 inactivation. Though this is unlikely, because vascular normalization is not known to completely reoxygenate tumors, it might happen. If it does happen, and realizing the benefits of HIF-1 inhibition requires that the benefits of vascular normalization be sacrificed, then we will see a less-then-additive effect of the proposed therapy combination. In this case, we would have at least learned something about vascular normalization and its effect on HIF-1 activation and radiation-induced ROS/RNS creation.

The potential pitfalls presented above represent some possible protocol failures and a spectrum of possible hypothesis failures. The protocol failures are less serious, because of the availability of multiple measurement methods for each quantity of interest. The hypothesis failures, though serious in terms of the consequences on potential treatment development, will still yield important information regarding the timing of multi-modal treatments and the interaction of HIF-1 with the tumor microenvironment. Regardless of its role in treatment, HIF-1 is an important regulator in tumor biology, and its various interactions must still be better understood.

4. CONCLUDING REMARKS

In this proposal, the combination of vascular normalization and HIF-1 blockade has been suggested as a potential means of sensitizing tumors to fractionated radiotherapy beyond the additive effect of each approach alone. This work will build on previous research that has identified vascular normalization and HIF-1 blockade as potential routes to radiosensitization. The hypothesis

behind the combination of the two approaches is that the reoxygenation caused by normalization will reduce the radioprotective effects that HIF-1 inhibition has on distal tumor cells. Furthermore, because the reoxygenated environment caused by vascular normalization might help to stabilize the active form of HIF-1 beyond what has been reported in the literature (for untreated tumors), post-irradiative HIF-1 inhibition is hypothesized to be an important factor in increasing the radiosensitizing effect of vascular normalization. It should be noted that because HIF-1 is, among other things, a VEGF transcription activator, DC101 (VEGFR-2 antibody) and YC-1 (HIF-1 inhibitior) overlap in their actions. However, the two treatments are different enough to justify their combination. VEGFR-2 antibody acts quickly on the current tumor microenvironment, while the HIF-1 inhibitor acts primarily after radiotherapy to prevent the transcription-mediated protective effects of HIF-1 activation. Also, VEGFR-2 antibody is an inhibitor of VEGF activity only, while HIF-1 inhibition prevents the activation of 60+ genes, including several genes involved in cell- and tumor-survival functions such as apoptosis, proliferation, and angiogenesis. As such, VEGFR-2 is used to alter the tumor microenvironment, while HIF-1 inhibition is used to block the tumor's response to radiation induced stress. Through their different but interacting mechanisms, VEGFR-2 and HIF-1 inhibitor should be able to enhance each other's radiosensitizing effects.

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[23] I don't like the way "I" sounds.